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ORIGINAL PAPER

Insulin regulation of growth hormone receptor gene expression: involvement of both the PI-3 kinase and MEK/ERK signaling pathways

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Abstract The mechanism(s) of insulin's effects on growth hormone receptor (GHR) gene expression are poorly understood. Using rat hepatoma cells, we have previously shown that insulin treatment reduces GHR mRNA and protein in a time- and concentration-dependent manner, at least in part via down-regulation of GHR transcription. The present study determines whether the phosphatidylinositol-3 kinase (PI-3 kinase) and mitogen activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways are involved in mediating these effects of insulin. Inhibition of the PI-3 kinase pathway partially blocked insulin's reduction of GHR mRNA, as did inhibition of the MEK/ERK pathway, resulting in higher GHR mRNA levels. Inhibition of both pathways was necessary to completely block insulin effects. Similar results were obtained for GHR protein. Collectively, these data suggest that insulin signaling via either the PI-3 kinase or MEK/ERK pathway may result in partial reduction of GHR gene expression, whereas signaling via both pathways may be required to achieve the full insulin effect.

Keywords Growth Hormone Receptor · Insulin · Signal Transduction

Abbreviations

ERK	Extracellular signal-regulated kinase
GH	Growth hormone
GHR	Growth hormone receptor
MEK	Mitogen activated protein kinase kinase
PI-3 kinase	Phosphatidylinositol-3 kinase

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Introduction

Insulin exerts profound effects on a variety of cellular processes. In addition to its direct effects on metabolism, insulin also serves as a potent regulator of gene expression. Insulin regulates the expression of an increasingly large number of genes whose proteins have a variety of functions [1, 2]. Several studies suggest that insulin regulates growth hormone receptor (GHR) gene expression [3–5]. Proper expression of the GHR is required for growth hormone (GH) to signal and elicit its biological effects. Hence, inhibition of GHR expression by insulin may have profound effects on cellular responsiveness to GH.

The role of insulin in regulating expression of the GHR remains unclear. This is mainly due to a poor understanding of the factors which may be important in determining insulin's effects on GHR. Further complicating our understanding of insulin's regulation of GHR, is the lack of sufficient studies regarding the mechanism(s) involved. These studies are impossible to perform in primary hepatocytes since the isolation and culture of primary hepatocytes greatly down regulates GHR levels [6]. The confounding presence of other hormones and growth factors makes these studies difficult to perform in vivo as well. The rat H4IIE hepatoma cell line is an ideal model for the present studies, in that it is extremely sensitive to both insulin and GH, and has been used in several studies examining the effects of insulin and GH [3, 7–15]. The H4IIE cell line is a derivative of the minimal deviation

H35 hepatoma cell line, and expresses receptors that bind insulin and GH with a similar affinity to isolated rat hepatocytes and adipocytes. Additionally, similar to the liver, insulin in these cells regulates the activity, mRNA expression, and transcription of several gluconeogenic enzymes [1, 2, 10–12, 16]. We have recently shown that in rat H4IIE hepatoma cells, insulin potently regulates GHR expression by reducing GHR protein, GHR mRNA, and GHR transcription in a time- and concentration-dependent manner, which is maintained in the continuous presence of insulin [3, 9, 15, 17]. To further explain these effects of insulin, the present study asked which insulin-sensitive signaling pathways were used to regulate GHR expression.

The effects of insulin, including regulation of gene expression, are mediated by the binding to its cell-surface receptors located at target sites. Insulin binding to its receptor initiates a cascade of phosphorylation and dephosphorylation events that lead to the eventual recruitment and activation of several components that together comprise the insulin-signaling cascade [18–22]. The phosphatidylinositol-3 kinase (PI-3 kinase) and mitogen activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways are integral components of the insulin-signaling cascade, and are known to be involved in mediating many of the effects of insulin, including regulation of hepatic gene expression [10, 12, 23–25]. Using pharmacological inhibitors, we investigated the involvement of these pathways in the insulin-induced reduction of GHR mRNA and protein expression. Results of these studies indicate that inhibition of either the PI-3 kinase or MEK/ERK pathway partially blocks insulin's reduction of GHR mRNA and protein, and to achieve the full effect of insulin, signaling via both of these pathways may be required.

Results

Effect of insulin on GHR mRNA expression

Rat H4IIE hepatoma cells were treated with insulin for various times, or left untreated, cells were harvested and Northern blot analysis was performed as previously described [12, 26–28]. Insulin treatment after 2 h moderately reduced GHR mRNA levels (20%), and caused even greater reductions after 3, 4, and 5 h of 60, 70, and 75%, respectively (Fig. 1, also see Figs. 3b, and 4b, open bars, for quantification of this effect). A maximum reduction of GHR mRNA levels of approximately 80% was seen after 6 h, and this effect of insulin was maintained for up to 24 h in the continued presence of active insulin (please see [3, 9, 15, 17]). To verify equal loading, all Northern gels were stained with acridine orange, and equivalent levels of 28S rRNA was observed in all lanes of the Northern blots used in these studies (Fig. 1, bottom panel; also see Figs. 3a and 4a, bottom panels).

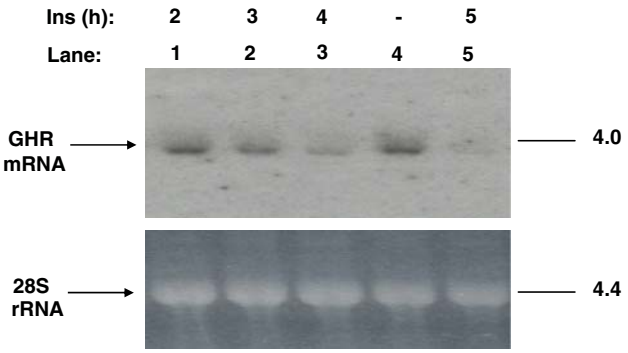

Fig. 1 Effect of insulin on GHR mRNA levels. H4IIE cells were serum-deprived and left untreated or treated with insulin for the times indicated. Following insulin treatment (10 nM), cells were harvested and subjected to Northern blot analysis using the rat GHR cDNA probe, as described in Materials and Methods. A representative Northern blot (where n is greater than 3) is presented with the acridine orange stained gel in the lower panel (see Figs. 3b and 4b for quantification). Insulin is denoted as "Ins"; time of treatment in hours is denoted as "h"; the dash on the upper panel denotes no treatment

Effect of insulin on activation of the MEK/ERK and PI-3 kinase pathways

To determine whether insulin activates the MEK/ERK and PI-3 kinase pathways in H4IIE cells, a time course of insulin treatment was performed. Insulin rapidly increased ERK1/2 phosphorylation by 5 min. By 4 h, the level of ERK1/2 phosphorylation was decreased, but remained elevated, indicating sustained activation of the MEK/ERK pathway (Fig. 2a). When cells were pretreated with the MEK inhibitor U0126, the increase in ERK1/2 phosphorylation was completely abolished. We have previously shown that insulin activates the MEK/ERK pathway in H4IIE cells, and that this activation is also blocked by another MEK inhibitor, PD98059 [10].

To examine insulin's activation of the PI-3 kinase pathway, phosphorylation of a downstream component of this pathway, Akt, was used as a marker. As with ERK1/2, insulin treatment for 5 min resulted in the rapid phosphorylation of Akt (Fig. 2b). By 4 h the level of Akt phosphorylation had decreased, but was still significantly elevated compared to untreated cells, indicating sustained activation of the PI-3 kinase pathway in the continuous presence of insulin (Fig. 2b). Phosphorylation of Akt, and therefore insulin activation of the PI-3 kinase pathway, was blocked by pretreatment with LY294002. This insulin-induced activation of the PI-3 kinase pathway may also be blocked by another pharmacological inhibitor, wortmannin (Fig. 2c). As with

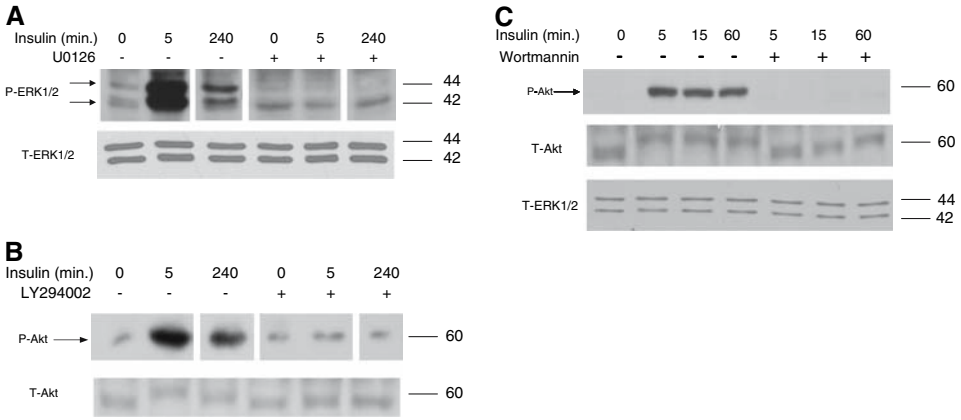

Fig. 2 Effect of insulin on activation of the MEK/ERK and PI-3 kinase pathways. H4IIE cells were treated with 10 nM insulin for the times indicated or were pretreated with U0126 (2 μM) or LY29004 (50 μM) or wortmannin (100 nM) prior to insulin treatment. Cells were then harvested and Western blot analysis was performed as described in Materials and Methods. (**a**) A representative Western blot of ERK1/2 phosphorylation and total ERK1/2 (where n is greater than 3) is presented. Time of treatment in minutes is denoted as "min". The upper panel was probed with anti-P-ERK1/2, and the lower panel was probed with total ERK1/2 antiserum to show no change in total protein. (**b** and **c**) Representative Western blots of Akt phosphorylation, total Akt and total ERK are presented. Total ERK1/2 probing was included as an additional control in (**c**) to show there were no changes in total protein levels

LY294002, pretreatment with wortmannin effectively blocked the insulin-induced phosphorylation of Akt at each time point examined. Since under the conditions used, the electrophoresed Akt band is retarded when it is phosphorylated and measured with a total Akt antibody (Fig. 2b and c), total ERK1/2 probing was included as an additional control to show there were no differences in total protein loading.

Effect of inhibition of the MEK/ERK pathway on insulin's reduction of GHR mRNA

Experiments were then performed to determine whether the insulin-induced reduction of GHR mRNA required insulin signaling through the MEK/ERK pathway. H4IIE cells were pretreated with the MEK inhibitor U0126 (25 μM) alone or U0126 for 30 min, followed by the addition of insulin (10 nM) for 2, 3, 4, or 5 h. Treatment with U0126 alone resulted in modest increases in GHR mRNA levels of 10–40%, but only the U0126 time-point at 5 h was significant compared to control (Fig. 3a,b, filled bars). This suggests that basal MEK-ERK activity suppresses GHR mRNA levels in cells not treated with insulin. Pretreatment with U0126 prior to the addition of insulin had a small, but significant effect (at 4 and 5 h) on blocking the reduction of GHR mRNA levels caused by insulin alone (Fig. 3a,b, hatched bars). In similar experiments, treatment with PD98059 resulted in a modest elevation of basal GHR mRNA and a slight reduction in the ability of insulin to reduce GHR mRNA levels (data not shown). These data suggest that signaling through the MEK/ERK pathway contributes only modestly to the reduction of GHR mRNA caused by insulin, and indicates that another insulin-sensitive signaling pathway may be involved.

Effect of inhibition of the PI-3 kinase pathway on insulin's reduction of GHR mRNA

To determine whether the reduction in GHR mRNA was mediated by insulin signaling through the PI-3 kinase pathway, experiments were performed with the pharmacological inhibitor LY294002. H4IIE cells were pretreated with LY294002 (50 μM) for 30 min, followed by the addition of insulin (10 nM) for 2, 3, 4, or 5 h. Other plates of cells were treated with LY294002 alone for 2.5, 3.5, 4.5, or 5.5 h. Treatment with LY294002 alone had little effect on GHR mRNA levels, resulting in only minor changes at each of the time points examined as compared to control (Fig. 4a,b, filled bars). Pretreatment with LY294002 prior to the addition of insulin had little effect on the minor reduction of GHR mRNA caused by insulin alone at 2 h, which was not significant. However, pretreatment with LY294002 partially blocked the reduction in GHR mRNA caused by insulin alone for 3, 4, and 5 h, and resulted in higher GHR mRNA levels (Fig. 4a,b, hatched bars). Specifically, pretreatment with LY294002 prior to the addition of insulin resulted in reductions of 24, 20, and 15% at the 3-, 4-, and 5-h time points respectively, compared to reductions of 60, 70, and 75% by insulin alone. Pretreatment with a combination of LY294002 plus U0126 prior to the addition of insulin totally blocked the reductions caused by insulin, and resulted in GHR mRNA levels similar to control (Fig. 4c).

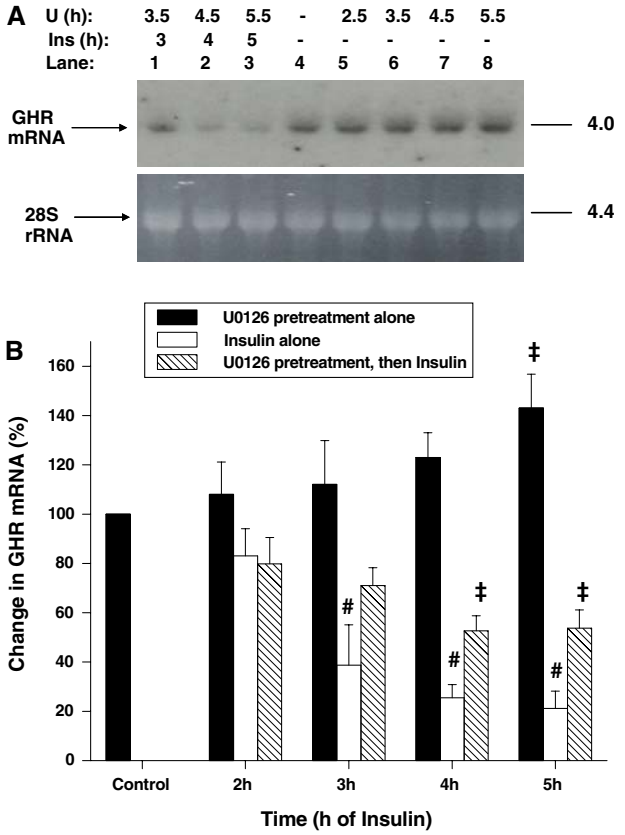

Fig. 3 Effect of inhibition of the MEK/ERK pathway on insulin's reduction of GHR mRNA. H4IIE cells were pretreated with 25 μM U0126 (abbreviated as U) for 30 min prior to the addition of 10 nM insulin (Ins) for the times indicated. Other cells were treated with DMSO alone (control) for the entire experimental period, or were treated with U0126 alone, or insulin alone for the times indicated; time of treatment in hours is denoted as "h"; a dash on the middle panel denotes no treatment. Northern blot analysis was performed as described in the legend for Fig. 1 and in the text. (**a**) A representative Northern blot is presented. (**b**) Densitometric analysis of autoradiographs from at least three similar experiments were performed to quantify the changes in GHR mRNA following times of various treatments. The data are expressed as the mean +/− S.E. The levels of GHR mRNA in control (DMSO-treated) cells were arbitrarily set to 100% within each experiment and the levels following times of various treatments are expressed as a percentage of that in control cells. All data were analyzed by ANOVA. The insulin alone 2-h time point is not significantly different from control, whereas the insulin alone 3, 4, and 5 h (#, $P < 0.001$) time points are significant. The U0126 alone 2-, 3-, and 4-h time points are not significantly different from control, whereas the U0126 alone 5-h (‡, $P < 0.05$) time point is significant. Pretreatment with U0126 prior to the addition of insulin for 2 and 3 h are not significantly different from the insulin alone 2- and 3-h time points. However, pretreatment with U0126 prior to the addition of insulin for 4 and 5 h (‡, $P < 0.05$) are significantly different from the insulin alone 4- and 5-h time points, respectively

Effect of inhibition of the MEK/ERK and PI-3 kinase pathways on insulin's reduction of GHR protein

We have previously shown that insulin treatment for 16 h significantly reduces immunoreactive GHR protein expression in H4IIE cells (data not shown, please see [3]). Experiments were performed to determine whether insulin's reduction of GHR protein was mediated by involvement of the MEK/ERK and PI-3 kinase pathways in a manner similar to that seen with GHR mRNA. H4IIE cells were pretreated with the MEK inhibitor PD98059 alone, or the PI-3 kinase inhibitor wortmannin alone, followed by Western blot analysis. Other plates of cells were treated with PD98059, wortmannin, or a combination of PD98059 plus wortmannin for 20 min, followed by the addition of insulin for 16 h. Pretreatment with PD98059 alone or wortmannin alone had little effect of GHR protein levels. Pretreatment with wortmannin prior to the addition of insulin partially blocked insulin's effects, and reduced GHR protein by 50% compared to a 74% reduction caused by insulin alone. Pretreatment with PD98059 prior to the addition of insulin also resulted in a partial blockade of insulin' s effects and reduced GHR protein by only 33% compared to a 74% reduction by insulin alone. Pretreatment with a combination of PD98059 plus wortmannin totally blocked insulin's effects and resulted in GHR protein levels similar to control (Fig. 5a,b). Expression of total STAT5b protein (control) was not altered in H4IIE cells in response to treatment with insulin, PD98059, or wortmannin.

Discussion

The present study further indicates that rat H4IIE hepatoma cells are a useful model for investigating the mechanism of insulin regulation of GHR gene expression, and suggests that the mechanism(s) may involve insulin-induced signaling through both the PI-3 kinase and MEK/ERK pathways. The H4IIE cells possess some activities found in rat hepatocytes and the liver, which suggest that effects presented here may be relevant to what occurs in normal hepatocytes. However, since these studies cannot be performed in isolated hepatocytes or in vivo, we must examine these effects of insulin in a model system, as used here. The PI-3 kinase and MEK/ERK pathways are integral components of the insulin-signaling cascade, and are known to be involved in mediating many of the actions of insulin including regulation of hepatic gene expression [10, 12, 23–25]. They were therefore considered as candidate pathways involved in insulin's regulation of GHR. Insulin and GH induce both the PI-3 kinase and MEK/ERK pathways in H4IIE cells, further indicating the suitability of H4IIE cells for studying the effects of insulin on GH signaling [3, 9, 10, 12, 13, 29].

The pharmacological inhibitor U0126 effectively blocked phosphorylation of both ERK1 and ERK2, thereby inhibiting insulin signaling through the MEK/ERK pathway.

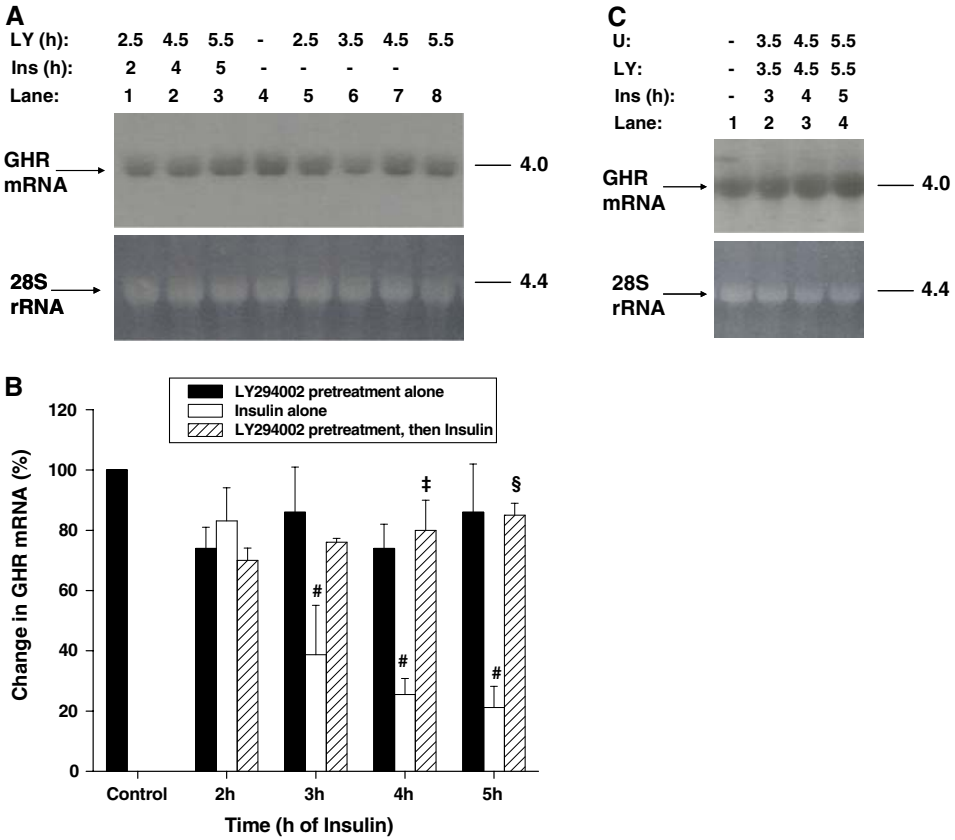

Fig. 4 Effect of inhibition of the PI-3 kinase pathway on insulin's reduction of GHR mRNA. H4IIE cells were pretreated with 50 μM LY294002 (LY) or a combination of LY294002 plus 25 μM U0126 (U) for 30 min prior to the addition of 10 nM insulin (Ins) for the times indicated. Other cells were treated with DMSO alone (control) for the entire experimental period, or were treated with LY294002 alone, or insulin alone for the times indicated; time of treatment in hours is denoted as "h"; a dash denotes no treatment. Northern blot analysis was performed as described in the legend for Fig. 1 and in the text. (**a**) A representative Northern blot is presented. (**b**) Densitometric analysis of autoradiographs from at least three similar experiments was performed to quantify the changes in GHR mRNA following times of various treatments. The data are expressed as the mean +/− S.E. The levels of GHR mRNA in control (DMSO-treated) cells were arbitrarily set to 100% within each experiment and the levels following times of various treatments are expressed as a percentage of that in control cells. The insulin alone 2-h time point is not significantly different from control, whereas the insulin alone 3-, 4-, and 5-h (#, $P < 0.001$) time points are significant. The LY294002 alone 2-, 3-, 4-, and 5-h time points are not significantly different from control. The pretreatment with LY294002 prior to the addition of insulin 2- and 3-h time points are not significantly different from the insulin alone 2- and 3-h time points, whereas the pretreatment with LY294002 prior to the addition of insulin for 4 and 5 h (‡, $P < 0.05$; §, $P < 0.01$) are significantly different from insulin alone for 4 and 5 h, respectively. (**c**) A representative Northern blot is presented

Inhibition of the MEK/ERK pathway with U0126 (or PD98059) only marginally blocked the reduction of GHR mRNA caused by insulin, suggesting that MEK/ERK pathway signaling may modestly contribute to insulin's reduction of GHR mRNA. Since treatment with U0126 (or PD98059) alone resulted in a gradual increase of GHR mRNA levels, these data also suggest that basal MEK/ERK activity may be important for maintaining basal levels of GHR mRNA expression.

The partial blockade of insulin's reduction of GHR mRNA which resulted from inhibition of the MEK/ERK pathway, suggested that signaling via another pathway may also be involved in mediating insulin's effects. Inhibition of the PI-3 kinase pathway with LY29004 partially blocked the reduction in GHR mRNA caused by insulin. Since treatment with LY29004 alone had little effect on GHR mRNA in the absence of insulin, this suggests that the higher levels of GHR mRNA were due primarily to blocking of insulin signaling through PI-3 kinase by LY29004, and not by alteration of basal levels of GHR expression. This finding thereby implicates the involvement of the PI-3 kinase pathway, possibly in combination with the MEK/ERK pathway, in mediating insulin's reduction of GHR mRNA. Hence, it is not surprising that inhibition of both pathways with the combination of U0126 and LY29004 prior to the addition of insulin, resulted in GHR levels that were indistinguishable from control (untreated cell) levels. Taken together, these data indicate

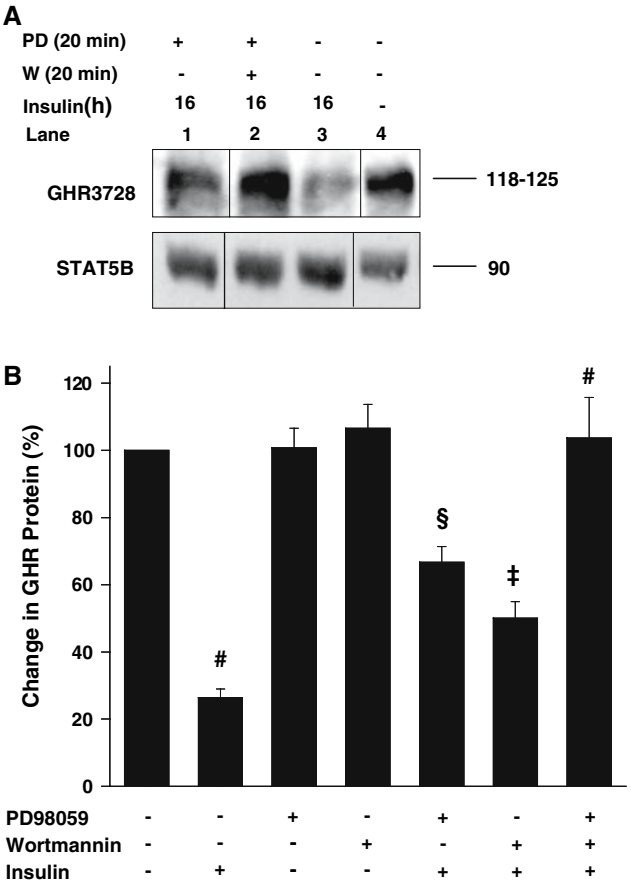

Fig. 5 Effect of inhibition of the MEK/ERK and PI-3 kinase pathways on insulin's reduction of GHR protein. H4IIE cells were pretreated with 10 μM PD98059 (PD), or a combination of PD98059 plus 25 nM wortmannin (W) for 20 min prior to the addition of insulin (10 nM) for the times indicated. Other cells were treated with DMSO alone (control) for the entire experimental period, or were treated with PD98059 alone, wortmannin alone (data not shown), or insulin alone for the times indicated; time of treatment in hours is denoted as "h"; time of treatment in minutes is denoted as "min"; a dash denotes no treatment. Western blot analysis was performed as described in the text. (**a**) A representative Western blot is presented. (**b**) Densitometric analysis of autoradiographs from at least three similar experiments were performed to quantify the changes in GHR protein following times of various treatments. The data are expressed as the mean +/− S.E. The levels of GHR protein in control (DMSO-treated) cells were arbitrarily set to 100% within each experiment and the levels following times of various treatments are expressed as a percentage of that in control cells. The insulin alone time point is significant compared to control (#, $P < 0.001$) None of the wortmannin or PD98059 alone time points are significant compared to control. The wortmannin for 20 min prior to the addition of insulin for 16 h (‡, $P < 0.05$), the PD98059 for 20 min prior to the addition of insulin for 16 h (§, $P < 0.01$) and the PD98059 plus wortmannin for 20 min prior to the addition of insulin for 16 h (#, $P < 0.001$) time points are significant compared to the insulin alone for 16-h time point

that insulin signaling through the MEK/ERK and even more through the PI-3 kinase pathway may partially reduce GHR mRNA expression, but signaling through both pathways may be required to achieve the full insulin effect.

Similar results were obtained when GHR protein was examined, further supporting the importance of both of these pathways in mediating insulin's effects on GHR expression. Due to the longer time course required for insulin's reduction of GHR protein (data shown at 16 h) versus GHR mRNA (3–5 h), the pharmacological inhibitors were used at lower concentrations in the experiments studying GHR protein expression. At the lower concentrations used, cellular viability was maintained over the extended time courses. The effects of inhibition of the PI-3 kinase and MEK/ERK pathways on GHR protein closely follows those obtained for GHR mRNA.

In summary, we present data indicating that insulin regulation of GHR gene expression in rat H4IIE cells involves both the PI-3 kinase and MEK/ERK signaling pathways. Insulin signaling via either pathway may result in partial reduction on GHR expression, but signaling via both pathways may be required to achieve the full effect of insulin. Therefore, these studies illustrate the involvement of two distinct signaling pathways acting in concert to regulate GHR expression. To our knowledge, these findings are the first to demonstrate such a mechanism to be involved in insulin regulation of the GHR gene. In combination with our earlier work, the data suggest that insulin reduction of GHR expression involves transcriptional down-regulation. Future questions involve determining the presence of one or multiple insulin-responsive elements within the GHR promoter.

Materials and methods

Materials

U0126 was purchased from Promega (Madison, WI), PD98059 from Cell Signaling Technology (Beverly, MA), LY294002 from BIOMOL (Plymouth Meeting, PA), and [alpha-^{32}P]dCTP from ICN (Irvine, CA). Fetal bovine serum, calf serum, and horse serum were purchased from Life Technologies, Inc. (Grand Island, NY). Wortmannin, porcine sodium-insulin, and other materials were purchased from Sigma (St. Louis, MO) and Fisher (Pittsburgh, PA) unless otherwise noted. The dosages of the pharmacological inhibitors used in these experiments were selected based on their IC50 (PD98059 = 4 μm and 50 μM; U0126 = 10–20 μM; LY294002 = 1.4–5 μM; Wortmannin = 2–4 μM) and experiments in our laboratory [10, 10–12, 30, 31].

Cell culture

Rat H4IIE cells (American Type Culture Collection, Rockville, MD) were plated at 1×10^6 and maintained at 37 °C and 5% CO_2 in Swim's 77 medium supplemented

with 5% horse serum, 3% newborn calf serum, and 2% fetal bovine serum since we have found these supplements to be optimal for growth [10, 11, 15]. At 70–80% confluence, cells were removed from serum and maintained in serum-free media for 18–24 h prior to experimental treatment to achieve optimal insulin sensitivity [3, 9, 15, 17].

Western blot analysis

Whole cell lysates were harvested following experimental treatments and Western Blot analysis was performed using anti-GHRcyt3728, P-ERK1/2, or P-Akt antiserum as previously described [3, 9, 10]. The anti-GHRcyt3728 antiserum, which is specific for the GHR protein, was kindly provided by Dr. Stuart J. Frank (University of Alabama at Birmingham) [3, 9, 13, 32].

RNA isolation

Total RNA was isolated following experimental treatments using the Ultraspec solution (Biotecx, Inc., Houston, TX). Briefly, cells were removed from media and lysed by triturating with the Ultraspec solution. Following complete dissociation of nucleoprotein complexes, isopropanol was added, and RNA was pelleted by centrifugation. The RNA pellet was extracted with phenol/chloroform:isoamyl alcohol and precipitated with ethanol. The concentration of RNA was measured by absorbance at 260 nm.

Northern transfer and analysis

Total RNA (10 μg) was electrophoresed on a 1.2% agarose, 2.2 M formaldehyde denaturing gel [12, 26–28]. The RNA was then transferred to Bright Star-Plus nylon membrane (Ambion, Inc., Austin, TX) by capillary action using the TurboBlotter System (Schleicher and Schuell, Inc., Keene, NH), and cross-linked by UV light (254–312 nm for 3-5 min). Membranes were preincubated in NorthernMax hybridization buffer (Ambion) for 2 h at 45°C, followed by addition of 1–5 $\times$ 10^6 cpm/ml of ^{32}P-labeled rat GHR cDNA for 16 h. The membranes were then washed and autoradiographed.

The cDNA for rat GHR for transcription rate and Northern analysis was kindly provided by Dr. Stuart J. Frank (University of Alabama at Birmingham). Labeling of the cDNAs was performed by random priming using the Prime-It II Kit (Stratagene, La Jolla, CA).

Statistical analysis

Densitometric data from autoradiographs were analyzed using ZERO-D scan from Scanalytics, Inc. (Fairfax, VA). All data were analyzed by ANOVA followed by a Dunnett post-test to compare all groups to the control group, using the InStat statistical program (version 3) from GraphPad Software, Inc. (San Diego, CA).

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